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Abstract

In the work outlined in the grant we propose to show that anti-Glut-1 antibodies can kill breast cancer cells in vivo. We have previously demonstrated that anti-glut-1 antibodies can kill breast cancer cell lines in vitro. Since the funding was initiated, we have further worked out the mechanisms by which anti-glut-1 antibodies kill cancer cells. We have since demonstrated that glut-1 antibodies, secondary to decreased glucose entry, reduces intracellular ATP generation consequently leading to increased pAMPK generation which acts as an energy sensor in the cell. pAMPK also has tumor suppressor properties Therefore leading to the up regulation of proapoptotic proteins BCL-XL and BAD and down regulation of anti-apoptotic proteins survivin and XIAP. Please see the attached figures. We have also shown that treatment with anti-glut-1 antibody decrease epithelial-mesenchymal transition (As seen in Figure 6). Since the P.I. has accepted a position as senior member at the Fox Chase Cancer Center, in Philadelphia PA further work will continue there. To initiate work on specific aim 1 and 2, the P. I. tested several commercially available antibodies and found these antibodies to be largely un-reliable in it's ability to detect the glut-1 antibody. Further testing continues. At the Fox Chase Cancer Center, there is core that generates antibodies. The P.I. has decided to generate our own antibodies which would be more reliable in its function and enable us to generate good data and pursue proof of principle of studies. The generation of these antibodies will be funded by the Fox Chase Cancer Center as part of the recruitment package given to the P.I. Once the new antibodies have been generated then we plan to proceed with specific aims one and two. It is anticipated that the antibodies will be available by Feb 09. Since the IACUC approval obtained at the Moffitt Cancer Center will be not be good any more, the P.I. will proceed to get the IACUC approval at the Fox Chase Cancer Center in Philadelphia PA which is anticipated to take approximately 3 months. Dr. Banerjee the Post doc assigned to this project is also moving with the P.I. and so the work will continue in this project seamlessly. The P.I. requests that the grant be transferred to Fox Chase Cancer Center so that work can continue

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Introduction:

Many human cancers display a high rate of anaerobic glycolysis. Glucose utilization by cancer cells is therefore greatly enhanced when compared to normal or benign tissues. Facilitative glucose uptake is achieved by transmembrane transporters, termed Glut-1-12, which are protein products of their respective GLUT genes. The Glut transporters differ in their kinetics and are tailored to the requirements of the cell type they serve, although more than one Glut may be expressed by a particular cell type.

A tumors' malignant potential is determined by its ability to generate energy and survive in hypoxic and acidotic environments. One of the key events that enable a tumor to survive hypoxia is its ability to generate energy by breaking down glucose in low oxygen tension environments by switching to anaerobic glycolysis. Persistent metabolism of glucose to lactate(i.e. anaerobic metabolism of glucose) even in aerobic conditions may be an adaptation to intermittent hypoxia in pre-malignant lesions. In comparison to aerobic glycolysis, anaerobic glycolysis is an inefficient method of breaking down glucose for energy *necessitating increased uptake of glucose by tumor cells*(38 (aerobic) versus 2 (anaerobic) molecules of ATP generated per molecule of glucose metabolized). *The increased intracellular uptake of glucose necessary to meet the glucose demands is facilitated by the membranous translocation of a high affinity Glucose Transporter (Glut-1) to the cell membrane.*

The Positron Emission Tomography (PET) scan utilizes this property for diagnostic purposes. Tumors preferentially upregulate a high affinity glucose transporter Glut-1 compared to normal cells that use other glucose transporters, particularly Glut-4. This difference between tumor and normal cells can be exploited for therapeutic gain. Autoradiographic studies of excised PET positive tumors with increased glucose uptake in viable cells near necrotic portions of tumor show that the membranous translocation of Glut-1 is responsible for that uptake. *Hence, tumor cell lines, in response to hypoxia, increase glucose uptake by up regulating membranous expression of the Glut-1 glucose transporter.*

Strategies that inhibit Glut-1 decrease proliferation and induce apoptosis in breast cancer cell lines. Aft et al investigated the effects of the anti-metabolite 2-deoxy-D-glucose(2-DG) on breast cancer cells *in vitro*. Treatment of human breast cancer cell lines with 2-DG resulted in a decrease of cell growth in a dose dependent manner. Other investigators transfected MKN45 cells with cDNA for antisense GLUT-1. Glucose transport was significantly decreased in cells with antisense GLUT-1 compared with wild-type cells or cells with vector alone. Tumor growth in the nude mice injected with antisense GLUT-1 expressing cells was significantly slower than in those with wild-type MKN45 cells. Conversely, increased differentiation of a breast cancer cell line T47D lead to a downregulation of membranous Glut-1. These results add to the increasing body of evidence that anti-GLUT-1 strategies can inhibit tumor growth. As part of this grant proposal we will continue this work in the *in vivo* model. We have also further worked out the mechanisms by which treatment with anti-glut-1 antibody kills cancer cells and these are outlined below.

Body

Methods

Cell culture and treatment

Breast Cancer line MDAMB-231 and mesothelioma cell line H2052 cells were cultured in DMEM and RPMI (Mediatech, Manassas VA) containing 10% FBS. Cells were treated in 2.5% serum containing media with anti-Glut-1 antibody twice a day [Alpha Diagnostic International, Inc San Antonio, TX (polyclonal) and Spring Bioscience, Fremont CA (monoclonal)] for 72 hours at a dose of 10ug/ml. LY294002 compound (Sigma Chemical Co, St. Louis, MO) and Compound C (Calbiochem, Gibbstown, NJ) was treated at dose of 10μM and 20μM respectively.

Tunnel assay

TUNEL assay was performed on the fixed cells using Dead End Colorimetric TUNEL system (Promega Biosciences Inc, San Luis Obispo, CA). Cells were plated in poly-D-lysine coated chamber slides at a density of 3,000 cells per well and then treated with anti-Glut-1 antibody and or LY294002 or compound C for 72 hr. Tunnel positive cells were visualized by microscopy and quantitated by counting 4 fields of 100 cells in quadruplicate. Data are presented as the percentage of tunnel positive cells out of the 100 cells counted.

Cell growth Inhibition Assay

To determine the inhibitory effect of anti-Glut-1 antibody in presence and or absence of drug on cell growth a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay will be used. Cells will be plated in 96-well plates and then cultured in medium with or without anti-Glut-1 antibody and after the treatment the percentage of viable cells in each well will be examined by MTT assay using spectrophotometer.

Lysate preparation and western blotting

Lysates from cells treated with anti-Glut-1 antibody and LY294002 were prepared by NP-40 lysis as described earlier^{11, 30} and 100ug protein was run on polyacrylamide-SDS gel and then immunoblotted with antibodies to pAKT, total AKT, AMPKα, total AMPKα, pBad, Bclxl, PTEN, PARP, Caspase-3, β-catenin, fibronectin, vimentin, total caspase-9, surviving, XIAP and Actin. Antibodies to pAKT, total AKT, AMPKα, total AMPKα, pBad, Bclxl, PTEN, PARP, Caspase-3 antibody were obtained from Cell Signaling Technology Inc, Danvers MA). β-catenin, fibronectin, Vimentin, total caspase-9, survivin were purchased from Santa Cruz Biotechnology, Santa Cruz CA). XIAP antibody is obtained from Stressgen Bioreagents Corporation, Victoria, BC). Antibody to Actin was purchased from Sigma Chemical Co, St Louis MO).

ATP assay

ATP assay was performed according to manufacturer protocol of Enliten ATP assay system bioluminescence detection kit. Briefly, 3000 cells/well were plated in 12 well plates and treated with antiglut-1 antibody for 72 hr. Following treatment, the cellular ATP was extracted (**Ref**) by adding 1ml of boiling water and cell suspension was made by repeated pipetting. Suspension of the cells was then transferred into a microcentrifuge tube for centrifugation (12,000g for 5 min at 4°C) and 10μl of the supernatant was used for bioluminescence measurement in turner biosystems 20/20ⁿ luminometer. The standard curve of ATP was obtained by serial dilution of 7μM ATP solution.

Transient transfection with small interfering RNAs

For the transient transfection with small interfering RNAs (siRNA), cells were plated in six-well plates. Glut-1 siRNA (Sigma Chemical Co, St Louis, MO) or control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) was transfected with Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells were used for ATP assay and Western blot analysis.

Migration Assay

The protective effect of anti-Glut-1 antibody on invasive ability of MDA-MB-231 cells was assayed according to the method reported before ²⁹. Briefly, the upper surface of the filters was precoated with collagen (100 g/filter). Matrigel was applied to the upper surface of the filters (50 µg/filter) and dried in a hood. These filters were placed in Boyden chambers. Cells were grown separately and treated with anti-Glut-1 antibody for 72 hr. Following treatment, cells were trypsinized and 5000 cells were plated in the upper chamber of the filter in media containing 0.1% bovine serum albumin (Sigma Chemical Co, St Louis, MO), Media containing 20% fetal bovine serum was placed in the lower well as an attractant and the chambers were incubated at 37°C for 18 hours. Non-migrating cells on the upper surface of the filters were removed after 18 hours by wiping with cotton swabs. The filters were processed first by fixing in methanol followed by staining with hematoxylin. The cells migrating on the other side of the filters were quantitated by counting three different fields under 40X magnification.

Key Research Accomplishments

Anti-Glut-1 antibody induces apoptosis in MDA-MB-231 cells. In figure 1, we show that MDA-MB-231 cells undergo apoptosis when treated with anti-Glut-1 antibody administered twice daily. Optimal apoptosis was seen after 72 hours, when compared to the control IgG-1 antibody (figure 1A). Therefore we selected twice a day instillation of anti-Glut-1 antibody for 72 hours for further experimentation. Apoptosis is demonstrated by PARP cleavage, decrease in total caspases 3 and 9 and cleavage of caspase 3 (figure 1B). Treatment with anti-Glut-1 antibody also leads to a downregulation of the Glut-1 transporter protein and mRNA. This is highlighted in figure 2.

Anti-Glut-1 antibody induces apoptosis through an Akt dependent mechanism. In figure 3A we show that treatment with the Glut-1 antibody decreases the expression of PI3-kinase (phosphoinositide 3-kinases) and phosphorylated Akt. There is no change in total Akt and PTEN (phosphatase and tensin homolog) protein expression. Apoptosis induced by the anti-Glut-1 antibody is partially reversed by co-treatment with PI3-kinase inhibitor, LY29002, suggesting that the apoptosis induced by anti-Glut-1 antibody is mediated through an Akt dependent mechanism, since Akt is downstream of PI3-Kinase. (figure 3).

Treatment with anti-Glut-1 antibody leads to decreased intracellular levels of ATP and increases phosphorylation of AMP kinase. After treatment with anti-glut-1 antibodies, intracellular levels of ATP are decreased in comparison to treatment with control antibodies (figure 4A). We postulate that treatment with anti-glut-1 antibody decreases the entry of glucose in to the cell, presumably by inducing a conformational change in the structure of the glucose transporter. In panel B of figure 4 we show that

treatment with anti-glut-1 antibodies increases the phosphorylation of the pro-apoptotic protein AMP kinase (5'AMP-activated protein kinase). Therefore anti-glut-1 antibodies promote apoptosis by down-regulating the activity of anti-apoptotic protein Akt and by upregulating the pro-apoptotic protein AMP kinase.

Anti-Glut-1 antibody treatment down regulates the expression of pro-survival proteins, XIAP and Survivin. In figure 5a, we show that the protein expression of anti-apoptotic proteins Bcl-xl and pBad are downregulated after treatment with anti-Glut-1 antibodies. This is accompanied by a downregulation of pro-survival proteins XIAP and survivin (figure 5b). Decreasing the protein levels of Bcl-xl and pBad and XIAP and survivin may make the cells vulnerable to treatment with chemotherapy, hormonal and targeted agents. This may partially explain why apoptosis induced by chemotherapy, hormonal and targeted agents is enhanced when these agents are co-treated with anti-Glut-1 antibody, as shown in our previous work

Anti-Glut-1 antibodies decrease the malignant potential of the treated cell lines by decreasing its ability to migrate and preventing its transition in to the mesenchymal phase.

In panel A of Figure 6, we show the treating MDA-MB-231 cells with anti-glut-1 antibody decreases its ability to migrate. In the in vivo system this presumably will decrease the ability of the cancer cells to migrate. Additionally, in panel B we show that the expression of mesenchymal phase proteins, fibronectin, focal adhesion kinase and vimentin, are decreased after treatment with the anti-glut-1 antibody. We saw no change in the expression of epithelial protein β -catenin and e-cadherin. This suggests that treatment with anti-glut-1 antibody prevents transition in to the mesenchymal phase where cells are resistant to treatment with chemotherapy and agents targeted to the epidermal growth factor receptor tyrosine kinase inhibitors.

Figure 1. Treatment with anti-Glut-1 antibody induces apoptosis in comparison to treatment with control IgG1 antibody as demonstrated by tissue culture panels in figure1(A)a and PARP cleavage, decrease in total caspase 3 and 9 and cleavage of caspase 3 in Figure 1B shows and . Optimal apoptosis is seen in at 72 hours (figure1A(b)).

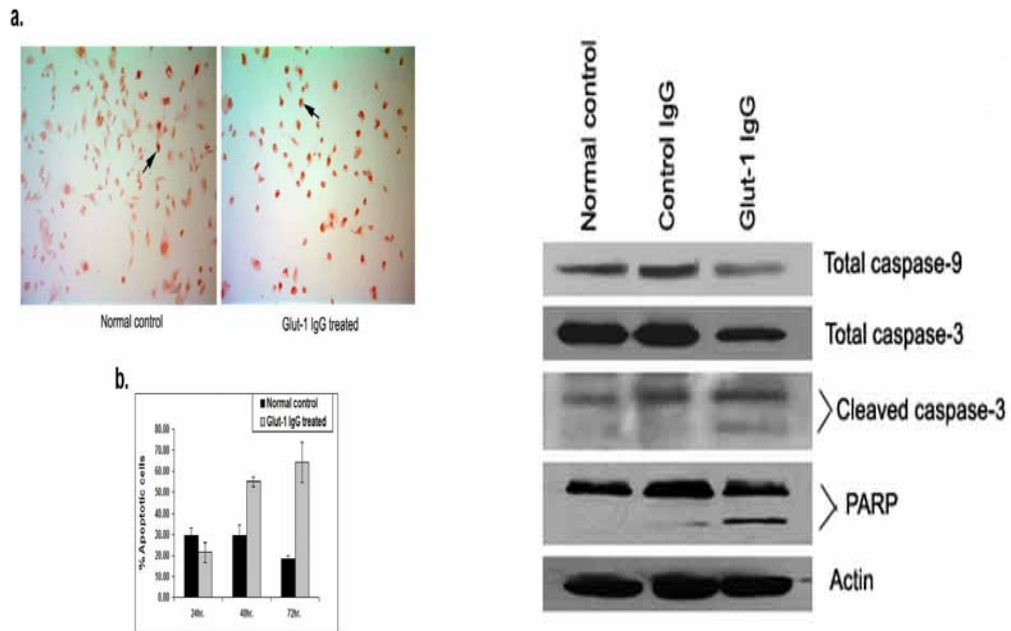


Figure 1A

Figure 1B

Figure 2. Treatment with anti-glut-1 antibody decreases Glut-1 protein expression as demonstrated by western blot (a) and Glut-1 mRNA expression as demonstrated by RT-PCR (b)



Figure 3. Treatment with anti-Glut-1 antibody decreases the protein expression of 85 α subunit of PI3Kinase, and pAkt, without changing total Akt and PTEN levels (figure 3A). In figure 3B, we show that the apoptosis induced by Glut-1 antibody is reversed by Akt inhibitor, LY29002 as demonstrated by TUNEL Assay (a) and PARP cleavage (b).

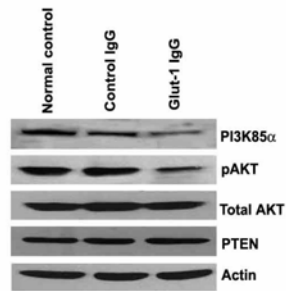


Figure 3A

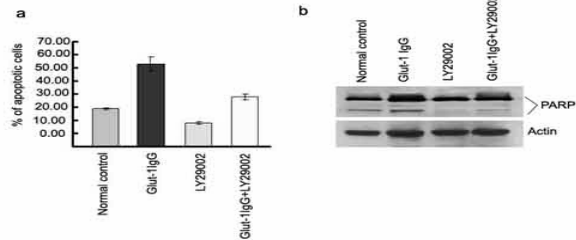


Figure 3B

Figure 4. Treatment with Glut-1 antibody decreases generation of ATP (figure 4A) and increase in pAMPK (figure 4B).

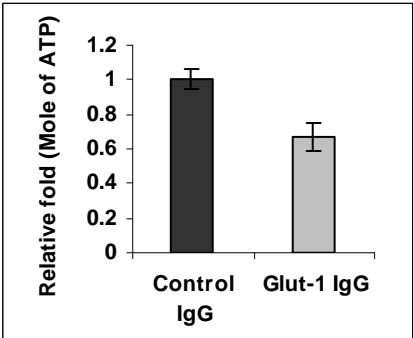


Figure 4A

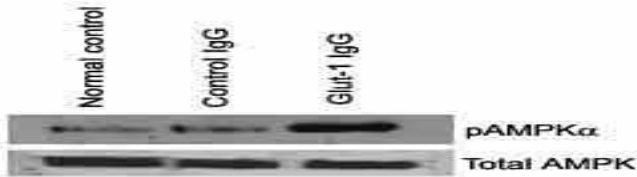


Figure 4B

Figure 5. Treatment with anti-Glut-1 antibody decreases the protein expression of anti-apoptotic proteins Bcl-xl and pBAD and pro-survival proteins XIAP and survivin.

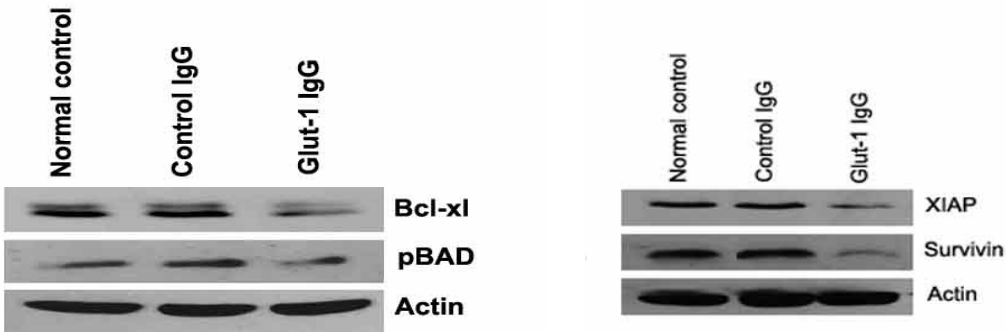


Figure 6. Treatment with anti-Glut-1 antibody decreases the ability of the treated MDA-MB-231 cells to invade (figure A). Decreased expression of mesenchymal phase proteins fibronectin, focal adhesion kinase (FAK) and vimentin in the anti-Glut-1 treated MDA-MB cells indicate decreased transition to the mesenchymal phase where the cells are less vulnerable to treatments with chemotherapy and epidermal growth factor-tyrosine kinase inhibitors (figure 6(B)). The expression of epithelial phase protein β-catenin is unchanged.

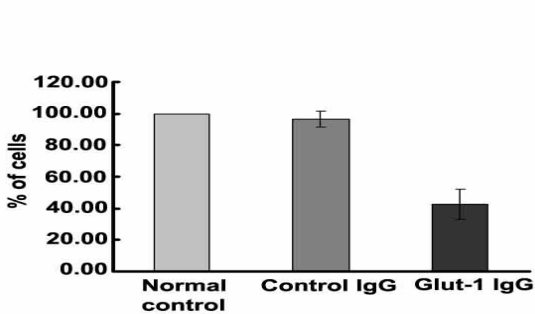


Figure 6A.

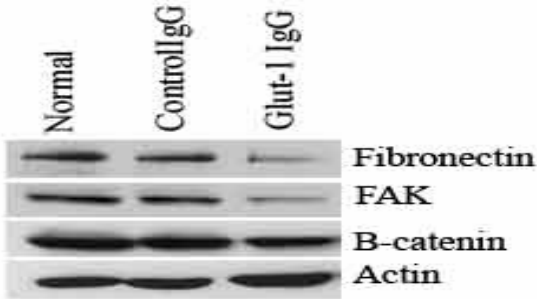


Figure 6B

Reportable Outcomes

We have demonstrated previously that the Glut-1 antibody induces apoptosis and reduces proliferation in the lung cancer and breast cancer cell lines. We additionally showed that it augments the apoptosis induced by chemotherapeutic agents like cisplatin and paclitaxel and targeted agents like gefitinib. In this report we delineate the mechanisms of anti-glut-1 antibody mediated apoptosis. Treatment with the antibody reduces phosphorylation of Akt which in itself is an anti-apoptotic protein. Additionally however, transcription of Glut-1 is mediated by Akt as evidenced by the presence of Akt responsive elements in its promoter. Therefore, decreasing pAkt will lead to a decrease in Glut-1 transcription which will lead to decreased Glut-1 mRNA levels and Glut-1 protein levels (figure 1b). The decrease in Akt mediated Glut-1 cell surface transporter expression will presumably lead to decreased glucose entry into the cells which consequently leads to decreased generation of ATP (figure 4). This decrease in generation of ATP, the primary source of energy utilized by the cells will impair the cells ability to carry out several critical energy dependent pro-survival functions, which we postulate is the reason why treatment with Glut-1 antibody impairs a cell's ability to invade.

The ability of the cell lines treated with anti-glut-1 antibody to survive and proliferate is diminished and its ability to undergo apoptosis is enhanced by several mechanisms. Pro-survival proteins XIAP and survivin are turned down and anti-apoptotic proteins i. e. bcl-xl and pBAD are down-regulated, as well. Additionally, the pAMPK α protein expression is increased. AMPK is a serine/threonine protein kinase, which serves as an energy sensor in all eukaryotic cell types. Published studies indicate that AMPK activation strongly suppresses cell proliferation in tumor cells. These actions of AMPK appear to be mediated through multiple mechanisms including regulation of the cell cycle and inhibition of protein synthesis. Activation of AMPK induces several tumor suppressor genes including LKB1, p53, TSC1 and TSC2, and overcomes growth factor signaling from a variety of stimuli including PI3K, Akt and ERK). Indeed the up-regulation of pAMPK may be one of the mechanisms of the down-regulation of pAkt. Down-regulation of pAkt in turn decreases the transcription of Glut-1 leading to reduced levels of the glut-1 protein which in turn decreases the entry of Glucose into the cell, which in turn reduces the intracellular ATP production.

Decreased intracellular ATP levels, decreased Glut-1 protein levels, up-regulation of pAMPK (and consequent up-regulation of tumor suppressors LKB1, p53, TSC1 and TSC2), down-regulation of pro-survival proteins XIAP and Survivin and down-regulation of anti-apoptotic proteins Bcl-xl and pBAD overall confer a less malignant phenotype to the anti-Glut-1 antibody treated cells, which in-turn manifests itself as decreased ability to invade and migrate and decreased transition to the mesenchymal phase from the epithelial phase and possibly even promote mesenchymal to epithelial transition. These properties also increase sensitivity to chemotherapy and targeted agents.

Conclusion

In conclusion, treatment with anti-Glut-1 antibody is not only capable of decreasing proliferation and inducing apoptosis by itself but also increases sensitivity to the treated anti-Glut-1 antibody treated cell lines to chemotherapy and targeted agents. The mechanism by which this is accomplished has become increasingly clear. Increased understanding of the mechanisms by which treatment with anti-glut-1 antibody induces cancer cell kill will enable us to develop rational combination therapeutics with Akt inhibitors and other drugs that inhibit proteins in this signaling pathway.

We now are proceeding with antibody experiments in mice. Before proceeding in mice several of the commercially available antibodies were tested in vitro systems and found to be unreliable in detecting the glut-1 protein. Meanwhile the P.I. and his lab is relocating to the Fox Chase Cancer Center in Philadelphia PA. The Fox Chase Cancer Center has core facility which

generates its own antibodies. This generally can be done in 3 months. The P.I. has decided to generate an antibody that binds to the extra-cellular domain of the antibody and test its reliability before proceeding with the in vivo experiments outlined in the DOD grant proposal. The monies to generate this grant will be funded by the Fox Chase Cancer Center that is part of the recruitment package offered to the P.I.

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Appendices:

None